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14. ABSTRACT

AIB1 (SRC3) belongs to the p160 family of steroid receptor coactivators including SRC-1 and SRC-2. AIB1 interacts with several nuclear receptors including estrogen and progesterone receptors in a ligand-dependent manner and enhances their transcriptional activity. AIB1 is amplified and/or overexpressed in approximately 30% of breast cancers and can increase the sensitivity of breast cancer cells to estrogen and to growth factor signaling. BRCA1 regulates cell cycle progression, apoptosis induction, transcription, and DNA repair. From 5-10% of total breast cancers are due to germ-line BRCA1 mutations that lead to a deficiency in the BRCA1 protein. We have observed that AIB1 can partially reverse BRCA1 mediated repression of ER-dependent transcriptional activity in breast cancer. This research will identify if there is a functional consequence of an interaction between AIB1 and BRCA1 in breast cancer.

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INTRODUCTION

The *AIB1* gene was initially identified to be amplified on a portion of human chromosome 20q that is frequently amplified in breast cancer (1). AIB1 (SRC-3) was later characterized as a nuclear receptor coactivator that belongs to the p160 family of steroid receptor coactivators including SRC-1 and SRC-2. Steroid receptor coactivators recruit other coactivators and the basal transcriptional machinery to nuclear receptors. AIB1 interacts with nuclear receptors including estrogen and progesterone receptors in ligand-dependent manner and enhances their transcriptional activity. The *AIB1* gene is amplified in 5-10% of human breast tumors and the mRNA is overexpressed in 31-64% of breast tumors (1-3). An isoform of AIB1, AIB1-Δ3, encodes a 130 kDa protein that lacks the amino-terminal bHLH/PAS dimerization domain and is a more active transcriptional coactivator of ERα and PR than full-length AIB1 (4). Also, AIB1 levels are limiting for both hormone (5-8) and insulin-like growth factor I (IGF-I) signaling responses in mammary cancer cells *in vitro* and for H-Ras-induced mammary tumorigenesis in mice (8).

Germ-line mutations of the breast cancer susceptibility gene *BRCA1* account for 5-10% of breast cancers. BRCA1 is involved in DNA repair, progression through the cell cycle, apoptosis, maintenance of DNA integrity, and regulation of transcription. BRCA1 has been shown to physically associate with transcriptional activators, repressors, DNA-binding transcription factors, and DNA repair factors. It has been reported that BRCA1 suppresses the transcriptional activity of estrogen and progesterone receptor (9, 10). The lack of wild-type *BRCA1* expression in mammary epithelium may lead to increased DNA damage and cellular proliferation. BRCA1 may suppress the proliferation of mammary epithelial cells by estrogen. Thus, the loss of the inhibitory activity of BRCA1 may lead to mammary carcinogenesis. It has been reported that BRCA1 interacts with SRC-1 (11), but there are no reports of an interaction of

AIB1 with BRCA1. Preliminary data in our lab showed that AIB1 and BRCA1 proteins interact in MCF-7 breast cancer cells. Since it has been reported that BRCA1 suppresses ERα activity in breast cancer cells, I assessed whether overexpression of AIB1 could reverse this effect. Also, preliminary evidence suggests that AIB1 can partially reverse the BRCA1-induced suppression of ERα transcriptional activity. We propose that the ratio of BRCA1 and AIB1 in the cell determines the response to estrogen. It could be that high BRCA1 levels in the cell leads to inhibition of AIB1's coactivator ability and decreased ERα activity, but when AIB1 is overexpressed or levels of BRCA1 decrease then AIB1 is free to coactivate ERα transcriptional activity. Increased estrogen-induced gene expression can lead to over-proliferation of breast epithelial tissue and eventually tumorigenesis. I want to determine if there is a functional consequence of AIB1 and BRCA1 in breast cancer in relation to hormone and growth factor signaling.

BODY

This annual report will address the progress for fulfilling the aims as outlined in **Task 1** and **Task 2** of the **Statement of Work** for grant W81XWh-05-1-0250. Section (a) of **Task 1** is to determine if AIB1 and BRCA1 protein interact directly in breast cancer cells and then define the region/s necessary for interaction with BRCA1. Preliminary experiments showed that AIB1 and BRCA1 protein interacted by co-immunoprecipitation using MCF-7 cellular lysate (Figure 1). In this experiment, BRCA1 was immunoprecipitated with a BRCA1 antibody (Ab2, Oncogene Science) and then AIB1 was detected by immunoblot with an anti-AIB1 antibody (BD Transduction). There was increased association of AIB1 with BRCA1 in this experiment and other similar experiments (Figure 1). However, experiments using a negative control (mouse IgG) suggested that a portion of the interaction was not specific because AIB1 was detected in

the mouse IgG immunoprecipitates. Another experiment was performed in 293T kidney cells, which are highly transfectable. 293T cells were transfected with Flag-epitope-tagged AIB1 and AIB1-Δ3 constructs using Fugene6. Lysates were immunoprecipitated with anti-Flag and immunoblotted for AIB1, BRCA1, and p300. The expression of AIB1 and AIB1-Δ3 was increased as indicated by the AIB1 immunoblot of the Flag immunoprecipitates (Figure 2). Interestingly, as indicated from the input samples, transfection of AIB1 and AIB1-Δ3 increased BRCA1 protein expression, thus suggesting that AIB1 may regulate BRCA1 expression in cells (Figure 2). BRCA1 was detected in the input samples, but there was no BRCA1 detected in the Flag immunoprecipitates (Figure 2). However, the p300 protein, which has been shown to interact directly with AIB1, was detected in the Flag-AIB1 immunoprecipitate (Figure 2). Therefore, AIB1 could interact with p300 but not BRCA1 in this experiment. The experiments so far for section (a) of **Task 1** suggest that AIB1 does not form a strong interaction with BRCA1. The question of whether AIB1 interacts with BRCA1 is inconclusive due to high non-specific interaction of AIB1 with the IgG immunoprecipitates as observed in some experiments.

Section (b) of **Task 1** is to determine if overexpression of BRCA1 in breast cancer cells is able to suppress the coactivator potential of AIB1 but not AIB1(BRCA1-) that does not bind to BRCA1. BRCA1 has been shown to suppress estrogen-dependent transcription in breast cancer cells (9). As part of the **Statement of Work**, it was determined if AIB1 can reverse BRCA1-dependent suppression of ER transcriptional activity. Preliminary data using MCF-7 breast cancer cells indicated that BRCA1 suppressed estrogen-stimulated estrogen receptor transcriptional activity and AIB1 could partially reverse this effect. However, these results were not reproducible in follow-up experiments.

For **Task 2**, it was determined if AIB1 and potentially if AIB1(BRCA1-) has any effect on IGF-1 or EGF-dependent biological responses, signaling, and gene expression. IGF-1 was not a strong inducer of cyclin D1 promoter activity. Therefore, HCC1937 (BRCA1-deficient)

and MCF-7 (BRCA1-wild type) breast cancer cells were tested for induction of cyclin D1 promoter activity by EGF and heregulin-β. Heregulin-β was a stronger inducer of cyclin D1 promoter activity in these cell lines. In HCC1937 and MCF-7 cells, induction of -1745 cyclin D1 promoter activity by heregulin-β was enhanced with overexpression of AIB1-Δ3 (Figure 3A and 3B). This suggests that enhancement of cyclin D1 promoter activity by AIB-Δ3 is independent of the status of BRCA1 in the cell.

Fulfilling the aims of **Task 2** was dependent on identifying a region of AIB1 that interacts with BRCA1. However, the ability of AIB1 to interact directly with BRCA1 was inconclusive. Therefore, it was necessary to compare EGF-dependent phenotypic effects in both BRCA-wild type and BRCA1-deficient breast cancer cells. It was determined if the expression level of AIB1 or BRCA1 affects the ability of cells to proliferate in response to EGF. MDA-MB-231 (BRCA1-wild type) breast cancer cells were tested because they proliferate in response to EGF. Initially, MDA-MB-231 cells were treated with AIB1 siRNA and then stimulated with EGF for 72 hr. A reduction of AIB1 protein levels in MDA-MB-231 cells (Figure 4) with AIB1 siRNA resulted in a significant inhibition of EGF-stimulated proliferation in comparison to control siRNA treated cells (Figure 4). Therefore, this demonstrates that cellular AIB1 levels are limiting for EGF-stimulated proliferation of breast cancer cells. It will be determined if there is a role for BRCA1 in EGF-stimulated proliferation of breast cancer cells. Since it was observed that there was a reduction in EGF-stimulated proliferation of AIB1 siRNA treated cells, it was determined if AIB1 knockdown affected EGFR levels or its autophosphorylation. Ligand-bound EGFR results in activation of tyrosine kinase activity and autophosphorylation of multiple intracellular tyrosine residues. As a result of AIB1 knockdown in MDA-MB-231 breast cancer cells, there was no change in EGFR levels (Figure 5A). However, there was a significant decrease in overall EGF-induced tyrosine phosphorylation of EGFR as detected with a

phosphotyrosine antibody (Figure 5A). This result was then verified with an antibody against phosphorylated EGFR on tyrosine 1173. Treatment of MDA-MB-231 cells with AIB1 siRNA resulted in decreased EGF-induced phosphorylation of EGFR on tyrosine 1173 (Figure 5B). It will be determined if the BRCA1 status of breast cancer cells affects EGF-induced activation of EGFR. Interestingly, it was previously shown that BRCA1 is phosphorylated through growth factor signaling (12). It is possible that AIB1 could regulate BRCA1 function through control of EGFR signaling.

Figure 1

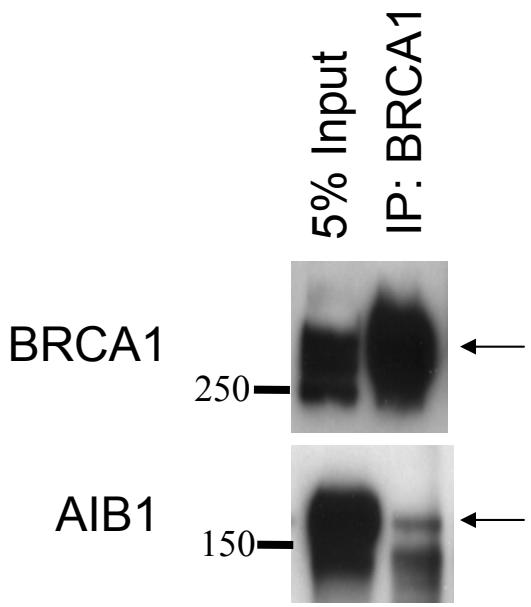

Figure 1. **Co-immunoprecipitation of AIB1 with BRCA1 in MCF-7 breast cancer cells**.
Whole cell lysate was immunoprecipitated with a monoclonal BRCA1 antibody (AB2, Oncogene Science) and then BRCA1 and AIB1 were detected with anti-BRCA1 (AB2) and anti-AIB1 (BD Transduction) antibodies.

Figure 2

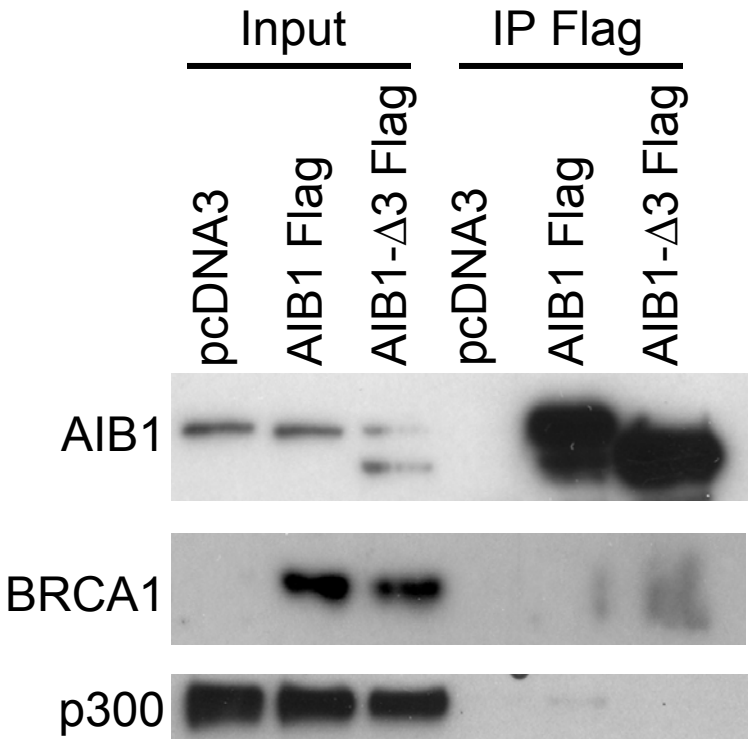

Figure 2. **Determine binding of BRCA1 with overexpressed AIB1 and AIB1-Δ3 in 293T cells.**
293T cells were transfected with either pcDNA3, AIB1-Flag, or AIB1-Δ3-Flag plasmid using Fugene 6 (Roche). Whole cell lysates were immunoprecipitated with anti-Flag (M2, Sigma) and then immunoblotted with either AIB1, BRCA1, or p300.

Figure 3

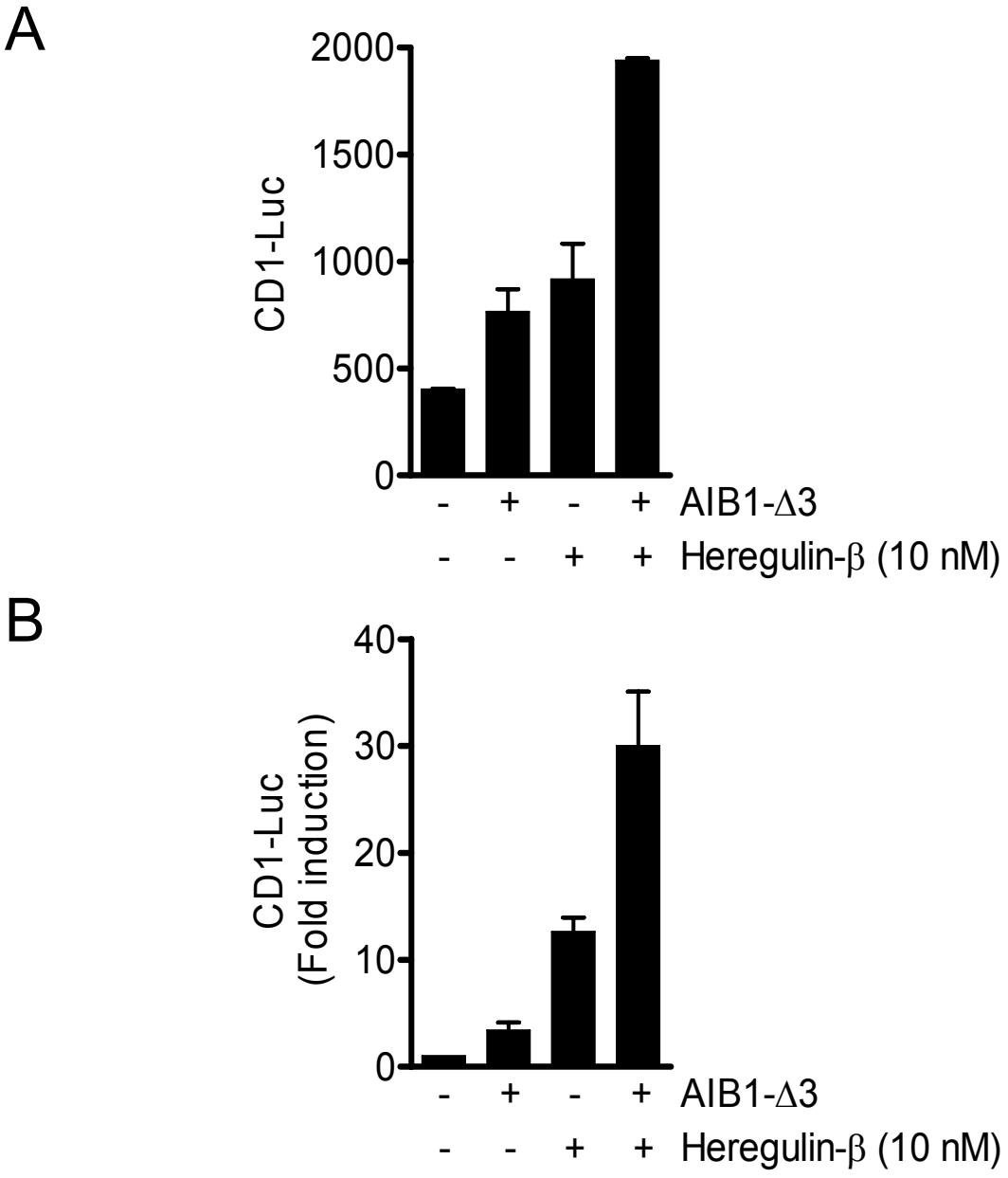

Figure 3. **AIB1-Δ3 enhances growth factor induced activation of the cyclin D1 promoter in BRCA1-deficient HCC1937 cells and BRCA1-wild type MCF-7 cells**. (A) HCC1937 and (B) MCF-7 cells were plated in IMEM containing 10% fetal bovine serum. After the cells were attached, the media was changed to serum-free IMEM. The cells were transfected with a -1745 cyclin D1 promoter-luciferase (CD1-Luc) reporter construct. After 24 hr of transfection, 10 nM of heregulin-β was added to the cells. The cells were lysed after 24 hr and luciferase activity was measured. The data from panel A represents a single experiment from triplicate samples. The data from panel B is from 5 separate experiments with 5 replicates per treatment and is represented as fold induction.

Figure 4

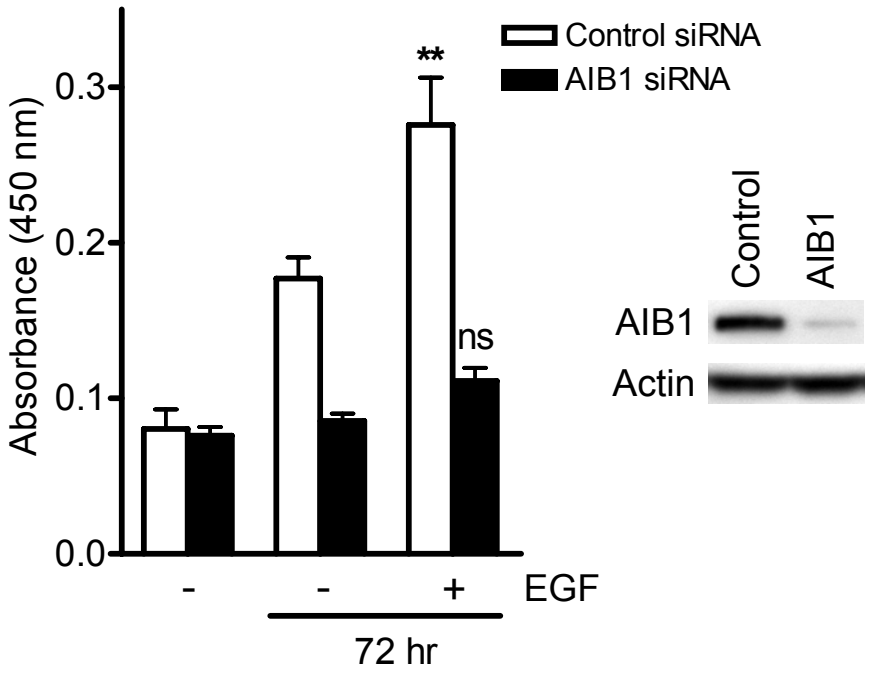

Figure 4. **AIB1 regulates EGF-stimulated proliferation of MDA-MB-231 breast cancer cells**.
MDA-MB-231 cells were transfected with either control siRNA (white bars) or AIB1 siRNA (black bars) for 24 hr, seeded into 96-well plates at a density of 2,500 cells/well and then serum-starved for 24 hr followed by EGF (50 ng/mL) treatment for 72 hr. Cell proliferation was measured using the WST-1 assay. The inset shows the level of AIB1 protein expression by Western blot analysis after 4 days of siRNA treatment. The columns represent the mean ± SD of triplicate values from four independent experiments, ** $P<0.001$ (one-way ANOVA) relative to control siRNA results for each respective treatment group.

Figure 5

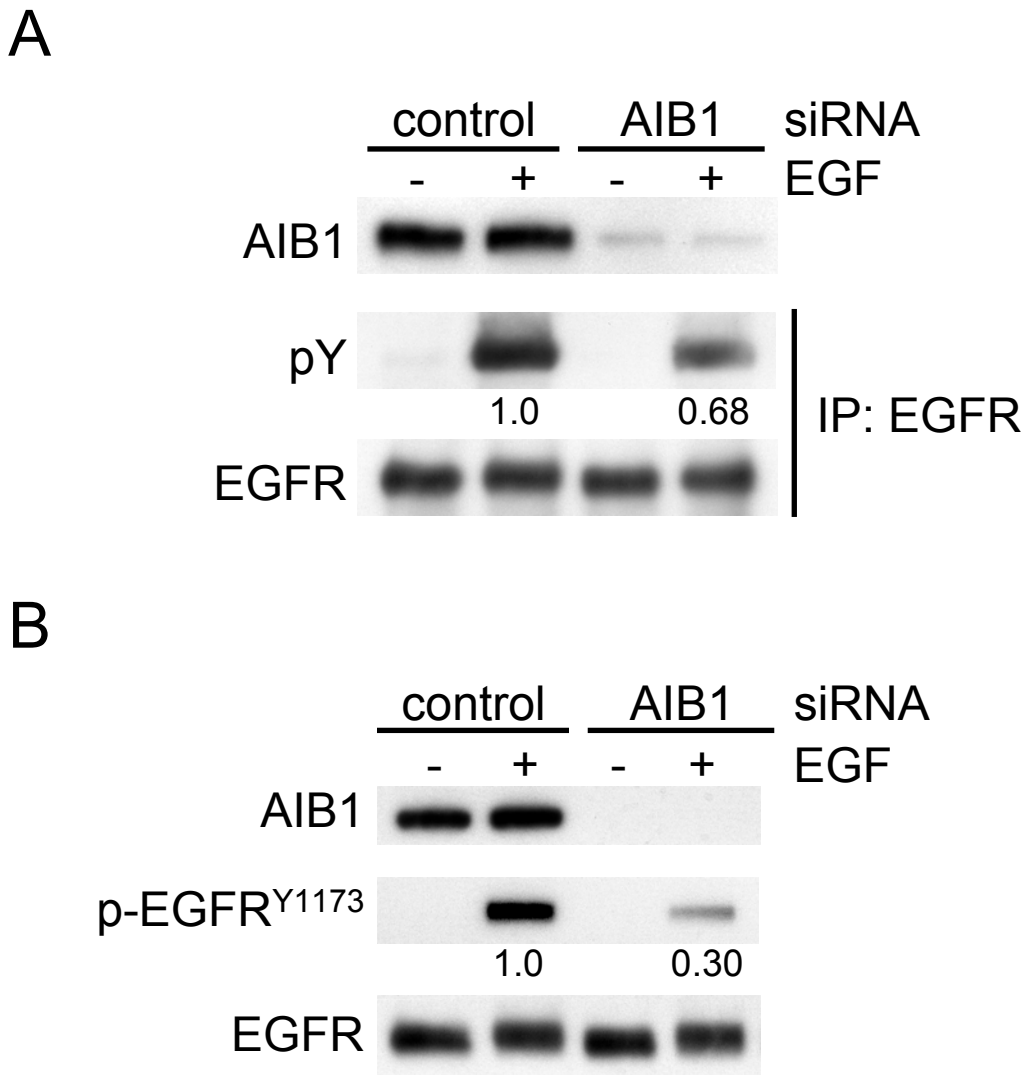

Figure 5. **Knockdown of AIB1 with AIB1 siRNA results in decreased phosphorylation of the EGF receptor in MDA-MB-231 breast cancer cells**. (A) MDA-MB-231 cells were treated with either control or AIB1 siRNA for 24 hr, serum-starved for 24 hr, and then stimulated with 50 ng/ml of EGF for 10 min. Whole cell lysates were immunoprecipitated with anti-EGFR (528, Santa Cruz) and then immunoprecipitates were immunoblotted with either anti-phosphotyrosine (4G10, Millipore) or anti-EGFR (1005, Santa Cruz). AIB1 levels were detected with anti-AIB1 (BD Transduction). (B) MDA-MB-231 cells were treated as in panel A. Whole cell lysates were immunoblotted for phosphorylated EGFR (p-EGFR^{Y1173}) (Cell Signaling) and total EGFR (1005, Santa Cruz).

KEY RESEARCH ACCOMPLISHMENTS

- Growth factor induced cyclin D1 promoter activity is enhanced in both BRCA1-wild type and BRCA1-deficient breast cancer cells.
- A reduction in AIB1 expression inhibits the proliferation of BRCA1-wild type breast cancer cells.
- A reduction in AIB1 expression decreases EGF-induced activation of EGF receptor in BRCA1-wild type breast cancer cells.

REPORTABLE OUTCOMES

Degrees:

Ph. D. degree in Tumor Biology obtained April 2007 from Georgetown University.

Publications:

1. **Lahusen T**, Fereshteh M, Oh A, Wellstein A, Riegel AT (2007) EGFR tyrosine phosphorylation and signaling controlled by a nuclear receptor coactivator AIB1. (Submitted to Cancer Research March 2007)

2. Mani A, Oh A, Bowden ET, **Lahusen T**, Lorick KL, Weissman AM, Schlegel R, Wellstein A, Riegel AT (2006) E6AP mediates regulated proteosomal degradation of the nuclear receptor coactivator amplified in breast cancer 1/SRC-3 in immortalized cells. Cancer Research. 66 (17):8680-6.

Abstracts:

1. **Lahusen JT**, Wellstein A, Riegel AT. AIB1 regulates EGFR phosphorylation and activity in cancer. Biomedical Sciences Research Fair (2006). Georgetown University, Washington, D.C.

2. Oh A, Stoica GE, **Lahusen JT**, Wellstein A, Riegel AT. Functional role of tyrosine phosphorylated AIB1/ACTR. Abstract No. 243. Keystone Symposia 2006, Nuclear Receptor: Orphan Brothers. Banff, Alberta, Canada

CONCLUSIONS

From **Task 1**, it is unclear if AIB1 and BRCA1 interact due to high non-specific binding of AIB1 with the IgG immunoprecipitates. This is a technical limitation of the experiment. In addition, interaction of AIB1 and BRCA1 could not be detected in 293T cells transfected with Flag-tagged AIB1 and AIB1-Δ3. Also, as part of **Task 1**, preliminary experiments showed that AIB1 was able to partially reverse BRCA1-mediated suppression of ERα transcriptional activity in MCF-7 breast cancer cells. However, BRCA1 did not consistently suppress ERα transcriptional activity in repeat experiments. To address **Task 2**, AIB1-Δ3 and growth factors were shown to enhance cyclin D1 promoter activity, which could be used as a model system for testing the functional relationship of AIB1 and BRCA1 in growth factor-dependent gene expression. It was observed that induction of cyclin D1 promoter activity by both heregulin-β and AIB1-Δ3 was independent of the BRCA1 status of the cell. Additionally, AIB1 was shown to regulate EGF-stimulated proliferation of BRCA1-wild type breast cancer cells and activation of EGF receptor.

ABBREVIATIONS

AIB1- Amplified in breast cancer 1
bHLH- basic helix-loop-helix
BRCA1- Breast Cancer Susceptibility Protein 1
EGFR- Epidermal Growth Factor Receptor
EGF- Epidermal Growth Factor
ERα- Estrogen receptor alpha
IGF-1- Insulin like growth factor 1
PAS- Per-Arnt-Sim
PR- Progesterone receptor
siRNA- small-interfering RNA

SRC- Steroid Receptor Coactivator

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